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Loss of PTPN23 Promotes Proliferation and Epithelial-to-Mesenchymal Transition in Human Intestinal Cancer Cells

van der Lely, Lisa ; Häfliger, Janine ; Montalban-Arques, Ana ; Bähler, Katharina ; Schwarzfischer, Marlene ; Sabev, Max ; Gottier, Claudia ; Lang, Silvia ; Scharl, Michael ; Spalinger, Marianne R

Abstract: Background/Objectives Protein tyrosine phosphatase nonreceptor type 23 (PTPN23) has recently been associated with several human epithelial cancers via regulation of growth factor signaling. Colorectal carcinoma (CRC) is a leading cause for cancer-related death worldwide and is associated with aberrant epidermal (EGF) and vascular endothelial growth factor signaling. Here, we investigated whether PTPN23 might play a role in CRC. Methods Expression of PTPN23 was analyzed in CRC tissue by immunohistochemistry. PTPN23 was silenced in HT-29 cells to address the role of PTPN23 in EGF signaling, gene expression, and cell migration. Results PTPN23 silencing in HT-29 and Caco-2 intestinal epithelial cancer cells significantly enhanced activation of pro-oncogenic signaling molecules and genes promoting epithelial-to-mesenchymal transition (EMT) upon EGF treatment, while genes encoding tight junction proteins were significantly reduced. Conclusions Our data clearly indicate that loss of PTPN23 is associated with increased activation of pro-oncogenic signaling pathways and an enhanced ability of human intestinal cancer cells to undergo EMT. Taken together, these findings show that PTPN23 acts as a tumor suppressor gene in CRC.

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Loss of PTPN23 promotes proliferation and epithelial-to-mesenchymal transition in human intestinal cancer cells.

Lisa van der Lely¹, Janine Häfliger¹, Ana Montalban-Arques¹, Katharina Bähler¹, Marlene Schwarzfischer¹, Max Sabev¹, Claudia Gottier¹, Silvia Lang¹, Michael Scharl^{1,2}, Marianne R. Spalinger^{1*}

Running Head: PTPN23 regulates EGF-induced signaling in colon cancer cells

¹Department of Gastroenterology and Hepatology, University Hospital Zurich, and University of Zurich, Zurich, Switzerland; ²Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland

***Corresponding author:** Dr. Marianne R. Spalinger, Department of Gastroenterology and Hepatology, University Hospital Zurich, Sternwartstrasse 14, 8091 Zurich, Switzerland. Phone: +41-44-255-37 94, Fax: +41-44-255-9497, E-mail: marianne.spalinger@usz.ch

Key words: colorectal cancer, Protein tyrosine phosphatase, EGF receptor, EMT, growth factor signaling

Abstract

Background/objectives: Protein tyrosine phosphatase non-receptor type 23 (PTPN23) has recently been associated with several human epithelial cancers via regulation of growth factor signaling. Colorectal carcinoma (CRC) is a leading cause for cancer-related death worldwide and is associated with aberrant epidermal and vascular endothelial growth factor (EGF and VEGF) signaling. Here, we investigated whether PTPN23 might play a role in CRC.

Methods: Expression of PTPN23 was analyzed in CRC tissue by immunohistochemistry. PTPN23 was silenced in HT-29 cells to address the role of PTPN23 in EGF signaling, gene expression and cell migration.

Results: PTPN23 silencing in HT29 and Caco-2 intestinal epithelial cancer cells significantly enhanced activation of pro-oncogenic signaling molecules and genes promoting epithelial-to-mesenchymal transition (EMT) upon epidermal growth factor (EGF)-treatment, while genes encoding for tight junction proteins were significantly reduced.

Conclusions: Our data clearly indicate that loss of PTPN23 is associated with increased activation of pro-oncogenic signaling pathways and an enhanced ability of human intestinal cancer cells to undergo EMT. Taken together, these findings show that PTPN23 acts as a tumor-suppressive gene in CRC.

Introduction

Colorectal carcinoma (CRC) is the third most common cancer worldwide in both men and women. Up to 70-80% of CRC develop sporadic, whereby somatic mutations mediate progression through the adenoma-carcinoma sequence. [1] These mutations, such as genetic mutations in the oncogene KRAS, which is found in 40% of CRC patients, result in altered response to growth factor receptors, indicating that deregulated growth receptor signaling is crucially involved in CRC pathogenesis. Beside this, genetic syndromes such as the Hereditary Nonpolyposis Colorectal Cancer (HNPCC) syndrome, Familial Adenomatous Polyposis and the Peutz-Jeghers syndrome make in total up to 5.1% of CRC cases. [2] Further, life-style factors, including low fiber and high red meat diet, high body weight, alcohol, low physical activity and advanced age are well known risk factors for the development of CRC. [1] Most CRC cases are diagnosed in Western countries with an annual increase of incidence. [3] Whether the tumor has metastasized to lymph nodes (LN) or distant organs is a key factor in determining patient's survival. [4] More than 50% are diagnosed with metastasis at or beyond stage III of the American Joint Committee on Cancer (AJCC) tumor/node/metastases (TNM) staging system. In case of positive metastasis, 5 year survival rate drops from 60% to 10%. [5] CRC is the fourth most common cause of cancer-related death worldwide [3], and investigating risk factors and molecular mechanisms involved in CRC pathophysiology is an important task to identify novel therapeutic targets.

Cells that acquire invasive and metastatic characteristics by sporadic epi-genetic modifications, are typical for the malignant progression of CRC. One such process, which allows cells to obtain invasive and metastatic potential, is so-called epithelial to mesenchymal transition (EMT). During EMT, polarized epithelial cells lose their characteristics and undergo tight junction detachment, impairment of apical-basal polarity, and reorganization of cytoskeletal architecture, and thereby gain mesenchymal properties such as increased motility. Growth factors, inflammatory cytokines and hypoxia regulate EMT in cancer cells, allowing the malignant cells to adapt to the tumor microenvironment and to develop an invasive and metastatic phenotype. [6] Of note, targeting epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) signaling is a well-established therapeutic approach in CRC treatment.

Protein Tyrosine Phosphatase Non-Receptor Type 23 (PTPN23, also known as His domain containing tyrosine phosphatase [HD-PTP]) has recently been associated with mammary epithelial cancer

development via regulation of EMT and subsequent disruption of the E-cadherin/beta-catenin association. [7] PTPN23 is a member of the Protein Tyrosine Phosphatases (PTP) family, which removes phosphate groups from phosphorylated tyrosine residues on target proteins, including surface receptors and downstream signaling molecules. In that way, PTPs regulate signal transduction events involved in cell differentiation, proliferation, apoptosis, adhesion, motility, invasion and migration. [7,8] Further, PTPN23 regulates epidermal growth factor receptor (EGFR) cell surface expression in many cell types. [9] Of note, EGF and its receptor EGFR play an important role in wound repair and cell growth. [10] PTPN23 forms part of the endosomal sorting complexes required for transport (ESCRT) pathway, which mediates EGFR internalization and endosomal degradation. PTPN23 deletion in epithelial cervix carcinoma cells leads to a stabilization of EGFR on the cell surface likely resulting in increased downstream signaling pathway activation. [9]

The gene locus encoding PTPN23 is located on the vulnerable chromosomal region 3p21.3, a hot spot for deletion in human epithelial cancers, [11] including breast cancer and epithelial cervix carcinoma, but also testicular germ cell tumors, lung, skin, prostate, and esophagus cancer. [12] Although, the role of PTPN23 in the pathophysiology of some epithelial tumors has been described, its role in colon cancer cells has not been identified yet. Therefore, the aim of this study was to investigate the role of PTPN23 in human colonic HT29 and Caco-2 epithelial cancer cells.

Materials & Methods

Materials. The following TaqMan genotyping assays were obtained from Thermo Fisher (Waltham, Massachusetts): PTPN23 (Hs00394144_m1), CDH1 (Hs01023894_m1), ETS-1 (Hs00428293_m1), ITGB6 (Hs00168458_m1), EGFR (Hs01076078_m1), Occludin (Hs00170162_m1), Vimentin (Hs00185584_m1), Zonulin-1 (Hs01551861_m1), DKK1 (Hs00183740_m1), Snail (Hs00195591_m1). Antibodies against human phosphorylated (Tyr416)/total Src, EGFR, phosphorylated (Tyr397)/total FAK; phosphorylated (Ser473)/total Akt; phosphorylated (Tyr202/204) ERK; phosphorylated (Tyr705)/total STAT3; E-cadherin, and phosphorylated (Ser552)/total β -catenin were obtained from Cell Signaling Technologies (Danvers, Massachusetts). Anti-human PTPN23 and anti β -actin antibodies were obtained from Sigma Aldrich (St. Louis, Missouri), and anti-human total ERK antibody from Millipore (Burlington, MA).

Cell culture. Human colonic HT29 and Caco-2 epithelial cancer cells were obtained from DMSZ and cultured in Dulbecco's modified Eagle medium (DMEM, Life Technologies, Park Paisley, UK) supplemented with 0.1% non-essential amino-acids (NEAA, Life Technologies) and 10% fetal calf serum (FCS, PAN Biotech, Brunschwig, Switzerland). Cells were grown in a 10% CO₂ atmosphere and splitted using trypsin before seeding 1×10^6 cells per well on 12 well tissue culture plates.

siRNA transfection. Directly before siRNA transfections, the cell culture medium was changed to culture medium without FCS (SF-DMEM). Knockdown of PTPN23 was then achieved using three human PTPN23 Silencer pre-designed siRNA constructs from Life Technologies in a concentration of 5nmol per ml. For transfection, all three PTPN23-specific siRNA constructs were mixed with Lipofectamine RNAiMax (Life technologies, Carlsbad, CA) and incubated at room temperature for 20 min., before adding to the cell culture media (SF-DMEM). After 8 hours, medium was changed to DMEM supplemented with 0.1% MEM NEAA and 10% FCS and cells were cultured for another 24 hours before further treatment.

Cell activation. For treatment with epidermal growth factor (EGF, R&D) cell culture medium was changed to SF-DMEM in order to minimize interference of growth factors present in the FCS with the experiments. After 8 h, cells were stimulated with EGF (100ng/ml) during 30min for protein isolation or 24 hours for RNA isolation unless specified differently.

Cell lysates. After stimulation, the cells were washed twice with ice cold Dulbecco's Phosphate Buffer Saline (PBS, without calcium chloride and magnesium chloride, Sigma Aldrich) and lysed in the assay-specific buffer.

RNA isolation and RT-PCR. Isolation of total RNA was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration was measured using a NanoDrop (ND-1000 Spectrophotometer, Witec AG, Littau, Switzerland). Complementary DNA (cDNA) synthesis was performed using the HighScribe reverse transcriptase kit (Applied Biosystems, Thermo Fisher). Real-time PCR was performed using FAST qPCR master mix (Thermo Fisher Scientific) on a RT-PCR QuantStudio 6 Flex system. All measurements were performed in triplicates, human GAPD (GAPH, Endogenous control (VIC, MGB Probe), Applied Biosystems, Thermo Fisher) was used as endogenous control and results were analyzed using the $\Delta\Delta CT$ method. The real-time PCR contained an initial enzyme activation step (5 min, 95 °C) followed by 45 cycles consisting of a denaturing (95 °C, 15 sec) and an annealing/extending (60 °C, 1 min) step.

Protein isolation and Western blots. Proteins were isolated using Mammalian Protein Extraction Reagent (M-Per, Fisher Scientific) according to the manufacturer's instructions. Equal amounts of proteins from each lysate were loaded on polyacrylamide gels and after separation by gel-electrophoresis blotted onto nitrocellulose membranes. Membranes were blocked in a 1% BSA and 3% Milk containing blocking solution, and incubated over night in blocking solution with an appropriate concentration of primary antibody. Membranes were washed three times with washing buffer (Tris buffered saline containing 1% Tween 20 (1%TBST)) before incubation with HRP-coupled anti-rabbit secondary antibody (Lab Force, Santa Cruz, CA) for 2 hours. Immunoreactive proteins were detected with a Fusion Solo S imager (Vilber Lourmat, Witec AG, Littau, Switzerland) using a Western Blotting detection kit (Western Bright Sirius or ECL, Advansta, Menlo Park, CA).

Immunoprecipitation and detection of phosphorylated EGFR. For detection of EGFR phosphorylation, 150µg of protein was pre-cleared on a shaker with Sepharose G beads (GE healthcare, Little Chalfont, UK) for 30 minutes prior to incubation at 4 °C with 5µg anti-EGFR antibody over night. Antibody-bound protein was then precipitated using Sepharose G beads according to the manufacturer's instructions, washed three times in ice-cold PBS and precipitates heated up in 20 µl 1x loading buffer (Invitrogen, Carlsbad, CA) for 10 minutes and supernatants used for Western blots as

described above. To detect phosphorylated tyrosine residues, a HRP-labeled, mouse anti-phosphotyrosine antibody (PY20; Cell Signaling Technologies) was used.

Electric Cell-substrate Impedance Sensing (ECIS). ECIS, (Applied BioPhysics, Trogny NY, USA) is a real-time, impedance based method used to quantify cell behavior considering cell-cell contacts. 100'000 HT29 cells/well were seeded on two 1E/8W plates (IBIDI, Planegg, Germany) and grown until confluency for 5 days. siRNA transfection was performed as described above. Two days later, medium was changed to SF-DMEM 8h before cells were wounded by applying a current of 2000mA for 20sec, at a frequency of 60'000Hz. Cells were washed with PBS prior to treatment with EGF (100ng/ml). Resistance and capacitance measurements were collected in real time at 64'000Hz (capacitance) and 4'000Hz (resistance). Obtained values were normalized to the offset point at the time of wounding.

Flow cytometry. For flow cytometry, HT-29 cells were seeded in 24 well plates at a density of 0.5×10^6 cell/well and transfected with siRNA prior to EGF treatment as described above. At indicated time-points, cells were washed in ice-cold PBS, detached from the culture plate using 1% trypsin/EDTA for 5 minutes and washed once in FCS containing culture medium to stop surface protein degradation. Cells were then washed again with PBS and incubated on ice with rabbit anti human EGFR (Cell signaling) and the Zombie NIR live-death discriminator for 15 minutes. Cells were washed, incubated for 10 minutes with AlexaFluor647 labeled anti rabbit antibody (Cell Signaling) prior to fixation with the FoxP3 fixation/permeabilisation kit from eBioscience. Cells were then incubated for another 15 minutes with PE-TexasRed-coupled anti Ki67 antibody (BioLegend, San Diego, CA). After washing, cells were analyzed on a LSR Fortessa from BD. For AnnexinV/PI staining to discriminate apoptotic cells, cells were detached from the culture plate as described above, prior to incubation in Annexin binding buffer (10mM HEPES, 150mM NaCl, 2.5mM CaCl_2 in PBS, pH7.4) for 10 minutes. Cells were then stained with AnnexinV and PI (both from Thermo Fisher Scientific) for 10 minutes at room temperature, prior to washing in Annexin binding buffer and analysis on a LSR Fortessa from BD.

Statistical analysis. Data are normalized to the respective control and presented as mean \pm standard deviation (SD). Statistical analyses were conducted by ANOVA followed by Student-Newman-Keuls test or Man-Whitney U where appropriate. P values <0.05 were considered significant.

Results

EGF treatment induces PTPN23 expression in HT29 and Caco-2 cells.

In a first step to investigate a possible role for PTPN23 in the pathogenesis of CRC, we investigated whether EGF stimulation affects PTPN23 expression in human colonic cancer cell lines. For this aim, we treated the human colon cancer cell line HT29 with 100ng/ml EGF for up to 48h and analyzed PTPN23 mRNA and protein expression by real-time PCR and Western blotting, respectively. As shown in Fig. 1A, we found an increase of *PTPN23* mRNA level with significant differences after 8h and 24h EGF-stimulation ($P<0.05$) in HT-29 cells. Fig. 1B confirms a significant increase on protein level ($P<0.05$). These data show that EGF induces PTPN23 expression, which indicates that PTPN23 might be involved in regulating EGF-mediated signaling cascades in HT29 cells. Of note, other factors involved in tumor growth and progression, such as tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF) did also induce PTPN23 mRNA and protein expression (Fig. 1C+D). However, co-treatment of EGF with TNF or EGF with VEGF did not further enhance PTPN23 levels (Figure 1C+D). Of note, PTPN23 also promoted PTPN23 expression in a second colon cancer cell line, namely Caco-2 cells (Figure 1E+F).

Loss of PTPN23 results in decreased EGFR degradation upon EGF treatment.

In a next step, we investigated whether EGF treatment affects cell surface EGFR localization in HT29 and Caco-2 cells and whether PTPN23 is involved in degradation of EGFR. Therefore, we transfected HT29 and Caco-2 cells with PTPN23-specific or with control siRNA. 24 h after siRNA transfection, cells were stimulated with 100ng/ml EGF for the indicated time (Fig. 2A+B) and EGFR expression was analyzed by Western blotting. Fig. 2A+B shows a clear reduction of EGFR expression in HT29 and Caco-2 cells after EGF stimulation at all time points. Interestingly, PTPN23 knock-down lead to a significant increase of EGFR protein expression compared to control cells, independent of EGF stimulation ($P<0.01$). It has been demonstrated that loss of PTPN23 results in reduced internalization of EGFR in cervix carcinoma cells [9]. Therefore, we next analyzed surface expression of EGFR in HT29 cells upon silencing of PTPN23 and treatment with EGF for 30 min., 1 h, 2 h, and 4 h. As expected, EGF-treatment resulted in a clear reduction of EGFR surface expression already after 30 min (Fig. 2C). Further, silencing of PTPN23 affected degradation of EGFR from the cell surface and

lead to an increase of EGFR surface stabilization after 30min EGF stimulation in PTPN23 knock-down cells compared to control cells. These data demonstrate that in HT29 cells, EGFR surface expression underlies PTPN23 regulation. Nevertheless, we still see a reduction of EGFR surface expression in PTPN23 knock-down cells upon presence of EGF.

PTPN23 regulates Src, FAK, β -catenin and ERK activation.

Having shown that PTPN23 regulates EGFR degradation in HT29 cells, we investigated its effect on the phosphorylation status of EGFR. For this aim, we again transfected HT29 cells with PTPN23-specific siRNA, or non-targeting control siRNA. As demonstrated in Fig. 3A, treatment with PTPN23-specific siRNA resulted in a clear reduction of PTPN23 protein expression. Immunoprecipitation of EGFR and subsequent analysis of tyrosine phosphorylation revealed that tyrosine phosphorylation and thereby activation of EGFR was significantly increased in PTPN23 deficient cells compared to control cells ($P < 0.01$, Fig. 3B).

We further investigated whether EGFR downstream signaling pathways were differentially activated upon silencing of PTPN23. As shown in Fig. 3C and D, Src and focal adhesion kinase (FAK) phosphorylation upon EGF treatment are significantly higher in PTPN23 deficient cells compared to control cells (100ng/ml for 30 minutes, $P < 0.05$, $P < 0.01$). FAK is a cytoplasmic tyrosine kinase involved in cell migration. Further, we found significantly increased β -catenin phosphorylation in EGF treated PTPN23 deficient cells compared to control cells ($P < 0.05$, Fig. 3E), while E-cadherin protein levels were not affected in PTPN23 deficient cells (Fig. 3F). Extracellular-signal regulated kinase (ERK) phosphorylation was significantly increased in PTPN23 deficient cells compared to control cells after EGF stimulation ($P < 0.05$, Fig. 3G). On the other hand, surprisingly, silencing of PTPN23 did not affect EGF-induced Akt phosphorylation upon deletion of PTPN23.

Similar results were obtained with EGF-treated Caco-2 cells upon PTPN23 silencing: also in these cells, we observed enhanced levels of EGFR phosphorylation, as well as downstream Src, FAK, β -catenin, and ERK phosphorylation (Fig. 4). These data indicate that PTPN23 plays a role in pro-oncogenic signaling pathways induced upon EGFR activation.

PTPN23 knock-down leads to loss of tight junctions and gain of EMT on mRNA level.

In a next step, we addressed how silencing of PTPN23 affects mRNA expression of EGF-induced genes in HT-29 cells. As expected, treatment with PTPN23 siRNA resulted in an efficient reduction of *PTPN23* mRNA levels ($P < 0.01$, Fig. 5A). Of note, mRNA levels of EGFR did not show a significant difference when HT29 cells were transfected with PTPN23-specific siRNA.

Having demonstrated an increase of Src, FAK, β -catenin, and ERK activation (phosphorylation) at protein levels in PTPN23 deficient cells, we now investigated mRNA levels of their target genes. We found a significant increase in mRNA levels of the transcription factor SNAIL in non-stimulated PTPN23 deficient cells compared to control cells ($P < 0.01$, Fig. 5B). On the other hand, the β -catenin antagonist Dkk1[6] was significantly reduced in EGF stimulated PTPN23 competent cells and even further reduced upon loss of PTPN23 compared to cells expressing normal levels of PTPN23 ($P < 0.05$, Fig. 5B). We further investigated genes encoding for tight junction proteins such as *CDH1* (encoding E-cadherin), *OCN* (Occludin) and *ZO-1* (Zonulin 1). [6] Fig. 5C shows a significant reduction of *CDH1* and *ZO-1* levels in PTPN23 knock-down cells, which was further pronounced through EGF stimulation ($P < 0.05$). On the other hand, PTPN23 deletion had no significant impact on *OCN* mRNA levels. At the same time, we found significantly higher mRNA levels of genes that encode molecules characteristic for a mesenchymal cell phenotype, such as *VIM* and *ETS-1* ($p < 0.05$; Fig. 5D). Integrin beta 6 (*ITGB6*) and tumor suppressing gene *TP53* mRNA levels were not altered significantly (Fig. 5D+E). These data demonstrate a clear correlation between PTPN23 loss of function and an increase in EMT enhancing factors on mRNA level.

Again, similar results were obtained with Caco-2 cells, where we found reduced levels of markers for epithelial phenotype, including *CDH1*, *ZO1*, and *DKK1*, while factors associated with EMT, such as *VIM* and *ETS1* were increased in EGF-treated Caco-2 cells (Fig. 6).

Loss of PTPN23 leads to an increase in proliferation and migration and to a decrease in apoptosis.

Having demonstrated PTPN23 deficiency impacts pro-oncogenic signaling pathways and EMT-associated gene transcription events in human HT29 colon cancer cells, we next investigated migration and proliferation of HT29 cells upon loss of PTPN23. To investigate how loss of PTPN23

affects cell migration and wound closure, we seeded HT29 cells in Electric Cell-substrate Impedance Sensing (ECIS) plates where wound closure can be measured via capacitance measurement. 5 days after seeding, when cells were 80% confluent, PTPN23 was silenced using siRNA. Two days later a wound was induced in the epithelial layer prior to treatment with EGF (100ng/ml) and capacitance was measured for several hours. As shown in Fig. 7A, cells treated with PTPN23 specific siRNA grew back to confluency much faster than control cells, leading to a faster reduction of capacitance. Of note, EGF treatment further enhanced this effect. To confirm this finding, we next analyzed the amount of proliferating cells by flow cytometry using the proliferation marker Ki67. Compared to control cells, loss of PTPN23 lead to significantly more Ki67+ (proliferating) cells after two and four hours of EGF stimulation ($P < 0.05$, Fig. 7B+C). To further address whether deletion of PTPN23 affects apoptosis, we used Annexin V and Propidium Iodide (PI) in flow cytometry. The rate of late apoptosis and necroptosis was significantly decreased in PTPN23 deficient cells compared to control cells, but this was not affected by EGF stimulation ($P < 0.05$, Fig. 7D-F). These data indicate that PTPN23 deficiency leads to significant changes in human colonic epithelial cancer cell behavior including proliferation, migration, late apoptosis and necroptosis.

Discussion

In the present study, we demonstrate that EGF stimulation results in increased PTPN23 expression in human colonic HT29 and Caco-2 epithelial cancer cells and that PTPN23 in turn regulates EGF-induced signaling cascades, EMT, cell migration and proliferation. These observations suggest for the first time that PTPN23 might be involved in the pathogenesis of colorectal carcinoma.

Beside inflammatory cytokines and hypoxia, growth factors such as EGF have been demonstrated to be elevated in the tumor microenvironment. [6] Our results indicate that increased levels of PTPN23 upon EGF treatment might protect cells from abnormally regulated EMT through de-phosphorylation and thereby inactivation of EGF-induced signaling pathways. EGF treatment results in internalization and degradation of EGFR. A recent study showed a correlation of PTPN23 deficiency in epithelial cervix carcinoma cells and stabilization of EGFR on the cell surface. [9] There, Ali *et al* demonstrated that PTPN23 is part of the ESCRT pathway that mediates EGFR internalization and endosomal degradation. In their experiments, PTPN23 deletion lead to diminished release of EGFR from ESCRT-0 to ESCRT-III, likely resulting in increased EGFR downstream signaling pathway activation. [9] Interestingly, we also found a correlation of PTPN23 deficiency and EGFR surface stabilization in human HT29 cells, indicating that the role of PTPN23 as part of the ESCRT pathway is not cell type specific, but rather a universal mechanism.

We have also demonstrated that PTPN23 regulates several signaling molecules downstream of EGFR. A recent publication showed that knockout of PTPN23 in mammary epithelial cells was associated with enhanced activation and auto-phosphorylation of FYN, a member of the Src kinase family. [11] Enhanced FYN phosphorylation upon loss of PTPN23 leads to increased phosphorylation of the cytoplasmic segment of E-cadherin and Tyr142 of β -catenin, finally resulting in the disruption of the E-cadherin/ β -catenin association. [11] As a consequence, E-cadherin is internalized in early endosomes by endocytosis, and thereby elevated FYN activation results in impaired integrity of adherens junctions. β -catenin activation in turn increases mesenchymal protein expression and promotes EMT. [7] In our study, we demonstrate that knock-down of PTPN23 in HT29 and Caco-2 cells leads to similar results, with significantly enhanced Src phosphorylation upon deletion of PTPN23. Src is a protein tyrosine kinase (PTK) and one of the best-investigated pro-oncogenic molecules, which regulates major cancer promoting cellular mechanisms, including proliferation, differentiation and survival. [13] Apart from increased Src phosphorylation, silencing of PTPN23 also

promotes FAK activation. In its activated state, FAK forms a complex with Src and activates signaling pathways that promote cell migration and angiogenesis. [14] With ERK being a member of the mitogen-activated protein kinases (MAPK), another signaling molecule controlling cell growth, migration and apoptosis is significantly up-regulated in PTPN23 deficient HT29 and Caco-2 cells. [15]

A further link to cell migration upon loss of PTPN23 can be drawn when considering that mRNA levels of *CHD1*, which encodes for E-cadherin, are significantly reduced in PTPN23 deficient cells. E-cadherin is a trans-membrane glycoprotein that links epithelial cells together at adherens junctions and exerts a tumor suppressive function through growth inhibition, reduction of invasiveness, apoptosis, as well as differentiation, and thereby protects from EMT. Reduced E-cadherin expression is associated with poor prognosis and survival in various human cancers, such as breast cancer, and liver carcinoma. [16] Beside this, E-cadherin inhibits β -catenin and in this way dampens transcription of downstream target genes of the proliferative Wnt signaling pathway. [16] In line with a tumor suppressive role for PTPN23, we detected a significant increase of phosphorylated β -catenin protein levels in PTPN23 deficient human HT29 and Caco-2 cells. Due to its function in enhancing Wnt signaling, β -catenin can be regarded as a proto-oncogene [17] and elevation of β -catenin has been found in sporadic CRC [18] and other tumors. [17].

EMT is induced through different signaling pathways, one of them being the Wnt/ β -catenin pathway. Wnt signaling induces translocation of β -catenin to the nucleus, increases levels of SNAIL and vimentin, and suppresses the expression of E-cadherin. SNAIL is a transcription factor, which is early activated in EMT and thereby plays an important role in cancer development. Its overexpression is associated with invasiveness, metastasis and decreased survival of CRC. [6] Our findings clearly point towards increased potential for EMT in PTPN23-deficient colorectal carcinoma cells: Upon PTPN23 knockdown, HT29 and Caco-2 cells show a significant induction of *SNAIL*, while the Wnt/ β -catenin antagonist *Dkk1*, is significantly reduced. On the other hand, genes encoding for tight junction proteins, namely *CDH-1* and *OCN*, which are both regulated by *SNAIL* [6], and *ZO-1*, which interacts with Occludin to organize epithelial polarization [19], are significantly reduced. Further, genes that promote mesenchymal characteristics, such as *VIM* and *ETS-1*, are significantly increased upon silencing of PTPN23 – possibly indirectly via increased SNAIL expression/activity. Among these, increased *ETS-1* expression is of great interest, since its protein product, c-ets-1, contributes to cancer

cell invasiveness and neo-angiogenesis, and high c-ets-1 levels are associated with poor survival of cancer patients. [20]

Our data indicate that loss of PTPN23 is associated with increased activation of pro-oncogenic signaling pathways and enhanced ability of colonic epithelial cells to undergo EMT. EMT is associated with aberrant cell proliferation, reduced apoptosis, poor differentiation, increased motility, invasiveness, metastasis and neo-angiogenesis [6]. In line with this, PTPN23-deficient HT29 cells showed a clear increase of cell migration, a significant increase in proliferation and a significant decrease in late apoptosis and necroptosis.

Of interest, PTPN23-deficient cells showed reduced levels of apoptosis/necroptosis even without EGF-stimulation. Cell preparation for flow cytometry involves mechanical dissociation of cell-cell contacts to achieve single cell suspensions, resulting in cellular stress and low levels of apoptosis since IECs are sensitive to apoptosis once they lose cell-cell and extracellular matrix contacts[21], a mechanism called anoikis[22]. Reduced sensitivity to anoikis in PTPN23-knockdown cells further indicates that loss of PTPN23 promotes survival of transformed IEC when they undergo EMT and dissociate from the epithelium.

Taken together, our data show that PTPN23 acts as a tumor-suppressor gene in CRC. Presence of EGF results in enhanced PTPN23 expression in human colonic HT29 and Caco-2 epithelial cancer cells, leading to a significant down-regulation of pro-oncogenic downstream signaling pathways, such as Src, FAK, β -catenin and ERK. Upon loss of PTPN23, genes encoding for tight junction proteins are significantly reduced, while EMT enhancing genes are significantly up-regulated. Our data confirm functional changes in PTPN23 deficient human colonic HT29 epithelial cancer cell behavior, leading to significantly more proliferation, more migration and significantly less late apoptosis and necroptosis. This indicates that loss of PTPN23 might be involved in the transition from non-invasive tumor cells to cancer cells with metastatic potential. [23] Nevertheless, further studies are needed to address the *in vivo* role of PTPN23 during colon cancer development.

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All persons contributing to this work are listed as authors on the manuscript.

Ethics Statement

None of the studies include human subjects or animal research.

Author Contributions

LL: performed most experiments, analyzed and interpreted all data, wrote the first draft of the manuscript; JH, AMA, KB, MaSc, MaSa, CG, and SL conducted additional experiments; LL, MiS and MRS analysed and interpreted the data; MRS and MiS designed and supervised the study; all authors wrote, corrected and approved the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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Figure Legends

Figure 1. Epidermal growth factor (EGF) stimulation induces PTPN23 expression. A+B: HT29 cells were treated with 100ng/ml EGF for indicated time. **A)** Real-time PCR analysis shows PTPN23 mRNA expression normalized to GAPDH and untreated control cells. **B)** Representative pictures from Western blot analysis of PTPN23 protein and respective densitometric analysis. **C+D:** HT29 cells were treated with 100ng/ml EGF, 100ng/ml VEGF, 100ng/ml TNF or a combination of EGF+VEGF or EGF+TNF for 24 h **C)** Real-time PCR analysis shows PTPN23 mRNA expression normalized to GAPDH and untreated control cells. **D)** Representative pictures from Western blot analysis of PTPN23 protein and respective densitometric analysis. **E+F)** Caco-2 cells were treated with 100ng/ml EGF for indicated time. **E)** Real-time PCR analysis shows PTPN23 mRNA expression normalized to GAPDH and untreated control cells. **F)** Representative pictures from Western blot analysis of PTPN23 protein and respective densitometric analysis. Data are normalized to the average of untreated control group and β -actin. Asterisks indicate significant difference vs. untreated control cells (n=5; mean \pm SD; * p < 0.05, one-way ANOVA).

Figure 2. PTPN23 promotes EGFR degradation. HT29 (**A+C**) and Caco-2 (**B**) cells were transfected with either PTPN23-specific siRNA or non-targeting control siRNA, followed by EGF stimulation with 100ng/ml for the indicated times. **A+B)** Representative pictures from Western blot analysis and densitometric analysis show EGFR protein expression after 1 h, 2 h and 4 h of EGF stimulation, compared to untreated control cells. **C)** EGFR surface expression on HT29 cells after 30min, 1h and 2h of EGF stimulation compared to un-stimulated control cells was analyzed by flow cytometry. Data are representative for 3 independent experiments. Asterisks indicate significant difference vs. the relative control (n=3; mean \pm SD; * p < 0.05, ** p < 0.01, one-way ANOVA).

Figure 3. PTPN23 regulates phosphorylation and thereby activation of Src, FAK, β -catenin, and ERK in HT-29 cells. PTPN23-specific siRNA and non-targeting control siRNA transfected HT29 cells were treated with EGF (100ng/ml) for 30 min. Shown are representative pictures from Western blot analysis and the respective densitometric analysis of **A)** PTPN23; **B)** phosphorylated and total EGFR from EGFR immunoprecipitates; **C)** phospho- and total Src; **D)** phospho- and total FAK **E)** phospho-

and total β -catenin; **F**) E-cadherin; **G**) phospho- and total ERK protein; and **H**) phospho and total Akt. Data are normalized to β -actin and the untreated, control siRNA transfected cells. Asterisks indicate significant difference vs. the relative control (n=5; mean \pm SD; * $=p < 0.05$, ** $=p < 0.01$, one-way ANOVA).

Figure 4. PTPN23 regulates phosphorylation and thereby activation of Src, FAK, β -catenin, and ERK in Caco-2 cells. PTPN23-specific siRNA and non-targeting control siRNA transfected Caco-2 cells were treated with EGF (100ng/ml) for 30 min. Shown are representative pictures from Western blot analysis and the respective densitometric analysis of **A**) PTPN23; **B**) phosphorylated and total EGFR from EGFR immunoprecipitates; **C**) phospho- and total Src; **D**) phospho- and total FAK **E**) E-cadherin; and **F**) Representative phospho- and total ERK protein expression. Data are normalized to β -actin and the untreated, control siRNA transfected cells. Asterisks indicate significant difference vs. the relative control (n=3; mean \pm SD; * $=p < 0.05$, ** $=p < 0.01$, one-way ANOVA).

Figure 5. PTPN23 knock-down leads to loss of tight junctions and gain of epidermal to mesenchymal transition (EMT) on mRNA level in HT-29 cells. HT29 cells were transfected with either PTPN23-specific siRNA or non-targeting control siRNA before treatment with EGF (100ng/ml) for 24 hours. The graphs show **A**) mRNA levels of PTPN23 and EGFR; **B**) mRNA levels of SNAIL and Dkk1; **C**) mRNA expression of CDH-1, OCLN and ZO-1 **D**) mRNA expression of VIM, ETS-1 and ITGB6. All data are normalized to the untreated control group and GAPDH. Asterisks indicate significant difference vs. the relative control (n=5; mean \pm SD; * $=p < 0.05$, ** $=p < 0.01$, one-way ANOVA).

Figure 6. PTPN23 knock-down leads to loss of tight junctions and gain of epidermal to mesenchymal transition (EMT) on mRNA level in Caco-2 cells. Caco-2 cells were transfected with either PTPN23-specific siRNA or non-targeting control siRNA before treatment with EGF (100ng/ml) for 24 hours. The graphs show mRNA levels of **A**) *PTPN23*; **B**) *CDH1*; **C**) *ZO1* **D**) *DKK1*; **E**) *VIM*; and **F**) *ETS1*. All data are normalized to the untreated control group and GAPDH. Asterisks indicate

significant difference vs. the relative control (n=3; mean \pm SD; * $p < 0.05$, ** $p < 0.01$, one-way ANOVA).

Figure 7. Loss of PTPN23 leads to an increase in migration and proliferation and to a decrease in apoptosis. Transfection with either PTPN23-specific siRNA or non-targeting control siRNA was performed as described above. **A)** Wounding with ECIS was conducted prior to treatment with EGF (100ng/ml). ECIS measured capacitance over time. Data are shown in comparison to the untreated relative control group and capacitance at the time of wounding. The lines show averages from four independent measurements for each condition. **B)** HT-29 and **C)** Caco-2 cells were stimulated with EGF (100ng/ml) for either one, two or four hours. The percentage of Ki67+ (proliferating) cells was analyzed by flow cytometry. Data are presented as percentage of the untreated relative control group in densitometric analysis. **D-F)** The rate of early and late apoptosis and necroptosis was analyzed by flow cytometry using the markers Annexin V and Propidium Iodide (PI). The graph shows **D)** representative FACS dot plots and statistical analysis of **E)** HT-29 and **F)** Caco-2 cells. Asterisks indicate significant difference vs. the relative control (n=3; mean \pm SD; * $p < 0.05$, one-way ANOVA).